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FORM PTO-1390 (REV 10-95) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**		**ATTORNEY'S DOCKET NUMBER** 1321.2.29.1 **U.S. APPLICATION NO (If known, see 37 CFR 1.5)** 10/009254
INTERNATIONAL APPLICATION NO. PCT/US00/17082	**INTERNATIONAL FILING DATE** 21 June 2000	**PRIORITY DATE CLAIMED** 21 June 1999

TITLE OF INVENTION
ISOLATED GENES FROM VIRULENT GROUP B STREPTOCOCCUS AGALACTIAE

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U S C 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S C 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U S.C 371(c)(2))
   a ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2))
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U S C. 371(c)(3))
   a ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
   b ☐ have been transmitted by the International Bureau
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U S C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U S C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U S C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1 97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3.31 is included
13. ☐ A FIRST preliminary amendment
    ☐ A SECOND or SUBSEQUENT preliminary amendment
14. ☐ A substitute specification
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information. Certificate of Mailing

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U.S. APPLICATION NO (If known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/009254	PCT/US00/17082	1321.2.29.1

17. [x] The following fees are submitted:

				CALCULATIONS	(PTO USE ONLY)
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):					
Search Report has been prepared by the EPO or JPO .... **$ 890.00**					
International preliminary examination fee paid to USPTO (37 CFR 1 482) . **$ 710.00**					
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1 445(a)(2)). ... . **$ 740.00**					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$1040.00**					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .... **$ 100.00**					
ENTER APPROPRIATE BASIC FEE AMOUNT =				710.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	67	(above 20)	X $ 18.00	$846.00	
Independent Claims	9	(above 3)	X $ 84.00	$504.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$	
TOTAL OF ABOVE CALCULATIONS =				$2060 00	
Reduction of ½ for filing by small entity, if applicable.				$	
SUBTOTAL =				$1030.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1 492(f)). +				$	
TOTAL NATIONAL FEE =				$1030.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$1030.00	
				Amount to be refunded:	$
				charged	$

a. [X] A check in the amount of $1030.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. __________ in the amount of $_____________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-0763. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Form PTO-1390 (REV 10-95)

ISOLATED GENES FROM VIRULENT GROUP B *STREPTOCOCCUS AGALACTIAE*

1. FIELD OF THE INVENTION

The present invention relates to genes isolated from Group B streptococci ("GBS"). More specifically, the present invention relates to genes that are specific to virulent forms of GBS and methods of using such genes and their products for the diagnosis and treatment of GBS infections.

2. TECHNICAL BACKGROUND

Group B streptococci ("GBS") are a common cause of disease in newborns, pregnant women, and other persons. Common manifestations of these infections include bacteremia, pneumonia, meningitis, endocarditis, and osteoarticular infections. C.J. Baker & M.S. Edwards, *Group B Streptococcal Infections*, in *Infectious Disease of the Fetus and Newborn Infant*, 980-1054 (J.S. Remington & J.O. Klein, eds., 1995); P. Munoz et al., *Arch Int Med* **157**:213-216 (1997).

Nearly 3 out of every 1,000 children born are infected with an invasive form of GBS disease. While GBS disease is of great concern in neonates, GBS is also an important pathogen in the general population, in which the incidence of invasive GBS disease is nearly 8 in 100,000. Of these infections, the mortality rate can be as high as 30%.

During childbirth, GBS can pass from the mother to the newborn. By one estimate, up to 30% of pregnant women carry GBS at least temporarily in the vagina or rectum without symptoms. Infants born to these women become colonized with GBS during delivery. Baker & Edwards, *supra*. Aspiration of infected amniotic fluid or vaginal secretions allow GBS to gain access to the lungs. Adhesion to, and invasion of, respiratory epithelium and endothelium appear to be critical factors in early onset neonatal infection. Baker & Edwards, *supra*; C.E. Rubens et al., *J Inf Dis* **164**:320-330 (1991). Subsequent steps in infection, such as blood stream invasion and the establishment of metastatic local infections have not been clarified. The pathogenesis of neonatal infection occurring after the first week of life is also not well understood. Gastrointestinal colonization may be more important than a respiratory focus in late onset neonatal disease. Baker & Edwards, *supra*. Considerable evidence suggests that invasion of brain microvascular endothelial cells by GBS is the initial step in the pathogenesis of

meningitis. GBS are able to invade human brain microvascular endothelial cells and type III GBS, which are responsible for the majority of meningitis, accomplish this 2-6 times more efficiently than other serotypes. V. Nizet et al. *Infect Immun* **65**:5074-5081 (1997).

Because GBS is widely distributed among the population and is an important pathogen in newborns, pregnant women are commonly tested for GBS at 26 to 28 weeks of pregnancy. Much of GBS neonatal disease is preventable by administration of prophylactic antibiotics during labor to women who test positive or display known risk factors. However, these antibiotics programs do not prevent all GBS disease. The programs are deficient for a number of reasons. First, the programs can be inefficient. Second, it is difficult to ensure that all healthcare providers and patients comply with the testing and treatment. And finally, if new serotypes or antibiotic resistance emerges, the antibiotic programs may fail altogether. Currently available tests for GBS are inefficient. These tests may provide false negatives. Furthermore, the tests are not specific to virulent strains of GBS. Thus, antibiotic treatment may be given unnecessarily and add to the problem of antibiotic resistance. Although a vaccine would be advantageous, none are yet commercially available.

Traditionally, GBS have been divided into 9 serotypes according to the immunologic reactivity of the polysaccharide capsule. H.M. Blumberg et al., *J Inf Dis* **173**: 365-373 (1996). Serotype III GBS cause 60-70% of all infections and almost all meningitis. Baker & Edwards, *supra*. Type III GBS can be subdivided into three groups of related strains based on the analysis of restriction digest patterns (RDPs) produced by digestion of chromosomal DNA with *Hin*d III and *Sse*8387 I. Y. Nagano et al., *J Med Micro* **35**:297-303 (1991); S. Takahashi et al., *J Inf Dis* **177**:1116-1119 (1998). Figure 1 illustrates a comparison of *Hind* III and *Sse*8387 I RDP typing of 62 type III isolates from Salt Lake City, Utah and Tokyo, Japan. Isolates were classified into types based on the similarity of the restriction digest patterns produced by *Hind* III or *Sse*8387 I digestion of chromosomal DNA. The two methods divided the isolates into RDP types containing exactly the same isolates: III-3 contains isolates 1-41, II-2 contains isolates 42-59, and II-1 contains isolates 60-62.

Over 90% of invasive type III GBS neonatal disease in Tokyo, Japan and in Salt Lake City, Utah is caused by bacteria from one of three RDP types, termed RDP type III-3, while RDP type III-2 are significantly more likely to be isolated from vagina than from

blood or CSF. These results suggest that this genetically-related cluster of type III-3 GBS are more virulent than III-2 strains and could be responsible for the majority of invasive type III disease globally.

From the foregoing, it will be appreciated that it would be a significant advancement in the art to provide one or more markers that are specific to virulent type III-3 GBS. It would be a further advancement to provide a method to exploit these markers for clinical identification of virulent type III-3 GBS. It would be a further advancement to provide methods for producing vaccines against type III-3 GBS.

Such compositions and methods are disclosed herein.

3. BRIEF SUMMARY OF THE INVENTION

The present invention relates to markers specific to type III-3 GBS. These markers, the *spb1* and *spb2* gene products (SEQ ID NO: 2 and SEQ ID NO: 4, respectively), are encoded by the *spb1* (SEQ ID NO: 1) and *spb2* (SEQ ID NO: 3) genes. The invention also provides these genes and gene products in substantially purified form.

In certain other embodiments, the present invention relates to recombinant vectors which incorporate the *spb1* gene or other nucleic acid molecules that code for the *spb1* gene product. The recombinant vector may be a plasmid. In certain embodiments, the recombinant vector is a prokaryotic or eukaryotic expression vector. In certain preferred embodiments, the nucleic acid molecule is operably linked to a heterologous promoter and/or other expression control elements, such as heterologous enhancers and polyadenylation sequences.

In certain other embodiments, the present invention relates to recombinant vectors which incorporate the *spb2* gene or other nucleic acid molecules that code for the *spb2* gene product. The recombinant vector may be a plasmid. In certain embodiments, the recombinant vector is a prokaryotic or eukaryotic expression vector. In certain preferred embodiments, the nucleic acid molecule is operably linked to a heterologous promoter and/or other expression control elements.

The present invention also provides host cells comprising the *spb1* and/or *spb2* genes. In other embodiments, a host cell of the present invention comprises nucleic acid molecules that code for the *spb1* and/or *spb2* gene products. The host cell may be a prokaryotic or eukaryotic host cell.

The present invention also relates to diagnostic methods for determining whether a mammal is infected or colonized by virulent GBS. In certain embodiments, a diagnostic method comprises the steps of (1) collecting a bodily fluid or culture from the mammal and (2) analyzing the bodily fluid or culture for the presence of absence of one or more gene products specific to type III-3 GBS, wherein the presence of one or more gene products specific to type III-3 GBS indicates infection or colonization by virulent GBS. The mammal may be a human. Alternatively, the mammal may be a laboratory, domestic, or agricultural animal. The bodily fluid or culture may be any bodily fluid or culture that is typically analyzed for the presence of bacteria. For example, the bodily fluid or culture may be a vaginal or rectovaginal culture. The bodily fluid or culture may also be a throat culture. The bodily fluid or culture may also be an endotracheal tube aspirant, fluid from a brochioaveloar lavage, or tissue from a lung biopsy. In certain embodiments, the bodily fluid or culture is blood, serum, amniotic fluid, cerebrospinal fluid, or joint fluid. Other sources of material will be apparent to those of skill in the art. In certain embodiments, a diagnostic method of the present invention comprises analyzing a sample for the presence or absence of the *spb1* and/or *spb2* gene product(s).

In certain embodiments of a diagnostic method of the present invention, the polymerase chain reaction ("PCR") is used to identify the presence or absence of the *spb1* and/or *spb2* gene(s). In certain other embodiments, antibodies are used to identify the presence or absence of the *spb1* and/or *spb2* gene products. The antibodies may be monoclonal or polyclonal antibodies.

The present invention also relates to GBS vaccines. In certain embodiments, the present invention provides vaccines comprising the *spb1* gene product, i.e., a protein comprising the amino acid sequence of SEQ ID NO: 2. In certain other embodiments, a vaccine comprises the *spb2* gene product, i.e., a protein comprising the amino acid sequence of SEQ ID NO: 4. In certain preferred embodiments, a vaccine comprises both the *spb1* and *spb2* gene products. The vaccine may include an adjuvant, such as alum. In certain other embodiments, the *spb1* and/or *spb2* gene(s) may be introduced into a mammal using either naked DNA or other gene therapy techniques to induce an immune response against type III GBS.

The present invention further provides methods of immunizing a mammal against GBS infection. In certain embodiments, such methods comprise administering to the

mammal a vaccine comprising an immunologically effective amount of a recombinantly produced protein comprising the amino acid sequence of SEQ ID NO: 2. In certain other embodiments, a method of the present invention comprises administering to the mammal a vaccine comprising an immunologically effective amount of a recombinantly produced protein comprising the amino acid sequence of SEQ ID NO: 4. The vaccine may also contain a mixture of the spb1 and spb2 gene products. Vaccines used in the methods of the present invention may further comprise an adjuvant, such as alum.

These and other features and advantages of the present invention will become more fully apparent from the following detailed description.

4. SUMMARY OF THE DRAWINGS

Figure 1 illustrates a comparison of *Hin*dIII and *Sse*83871 RDP typing of 62 type III GBS isolates from Salt Lake City, Utah and Tokyo, Japan. Isolates were classified into types based on the similarity of the restriction digest patterns produced by *Hind*III or *Sse*83871 digestion of chromosomal DNA. The two methods divided isolates into RDP types containing exactly the same isolates: III-3 contains isolates 1 - 41, III-2 contains isolates 42 - 59, and III-1 contains isolates 60 - 62.

Figure 2 illustrates a dot blot hybridization of probe 1 with genomic DNA isolated from type III GBS. 10 μg of genomic DNA from each of 62 type III GBS strains was transferred to nylon membrane. Radiolabeled probe 1 hybridized with DNA from all III-3 strains (rows A - D) including the original type III-3 strain (well E1). The probe failed to hybridize with DNA from III-2 strains (F1 - F10, G1 - G7) including the original strain used in the subtraction hybridization (well E10) and III-1 strains (wells H1 - H3). The same pattern of hybridization was observed using clone 3 and 11 probes.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification and prevention of infections by virulent forms of GBS. The present invention also relates to isolated genes specific to type III-3 GBS. These genes, *spb1* and *spb2*, encode the *spb1* and *spb2* gene products.

The *spb1* and *spb2* genes or other nuclceic acid moleucles coding for the *spb1* or *spb2* gene products may be incorporated into a recombinant vector using methods known in the art. *See, e.g.*, 1-3 J. Sambrook et al., *Molecular Cloning: A Laboratory Manua*l

(2d ed. 1989). Recombinant vectors include any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, etc., that is capable of replication when associated with the proper control elements and that can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles.

A nucleic acid molecule of the present invention may be operably linked to expression control sequences, such as heterologous promoters. Examples include, but are not limited to, viral promoters such as the SV40 early promoter and the CMV immediate early promoter region, bacterial promoters, mammalian promoters, inducible promoters, synthetic promoters, hybrid promoters, and the like. Other expression control sequences are known in the art and include polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), and enhancers. These expression control sequences collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present in a recombinant vector, so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

Recombinant vectors can be constructed to include selectable markers. Suitable markers include genes which confer antibiotic resistance or sensitivity, or impart color, or change the antigenic characteristics when host cells which have been transfected with the recombinant vectors are grown in an appropriate selective medium. Suitable markers are known to those of skill in the art.

The discovery of type III-3 GBS-specific gene products will allow clinicians to diagnose and treat infection and colonization with virulent GBS. For example, hybridization-based assays may be used to determine whether a GBS isolate is type III-3. Figure 2 illustrates the results of hybridization assays with a III-3-specific probe. Likewise, PCR may be used to detect the presence or absence of either the *spb1* gene or the *spb2* gene (or both) in samples from patients. PCR methods are described generally in C.R. Newton & A. Graham, *PCR* (2nd. ed. 1997); *PCR: Essential Techniques* (J.F. Burke ed., 1996). Patients who are infected with type III-3 GBS may then receive appropriate antibiotic therapy.

Antibodies may also be used to detect the presence or absence of the *spb1* and/or *spb2* gene product(s). Methods for preparing both monoclonal and polyclonal antibodies are described in, e.g., E. Harlow & D. Lane, *Antibodies: A Laboratory Manual* (1988).

The present invention also relates to methods for producing type III-3 GBS vaccines. *See generally Vaccine Protocols* (A. Robinson, G.H. Farrar & C.N. Wiblin eds. 1996). In certain embodiments, the *spb1* and/or *spb2* gene product(s) may be used to immunize against GBS. These gene products may be produced in large quantities using techniques that are known in the art. For example, the appropriate gene or genes may be linked to a prokaryotic promoter and expressed in bacteria. The gene products may then be purified using conventional techniques and used to vaccinate at-risk individuals. Alternatively, the appropriate gene or genes may be linked to a eukaryotic promoter and enhancer (e.g., yeast, baculovirus, SV40, etc.) and expressed in an appropriate cell type. The gene products may then be purified using conventional techniques.

The *spb1* and/or *spb2* gene products, or immunogenic fragments thereof, may stimulate an immune response when administered to a host. Recombinantly produced proteins are especially desirable, as they can be produced in large amounts and purified. Furthermore, recombinantly produced proteins may be engineered to maximize desirable activities and to minimize unwanted effects.

The recombinantly produced *spb1* and/or *spb2* gene products may be used as carrier proteins for a polysaccharide-protein or oligosaccharide-protein conjugate vaccine. *See, e.g.*, R. Schneerson, et al., *Infect Immun* **60**:3528-3532 (1992) (describing a *Pneumococcus*-pertussis toxin conjugate vaccine). For example, *Haemophilus influenzae* B vaccines have been produced by conjugating a tetanus toxoid; a *Corynebacteriaum* toxin, CRM_{197} (which is a mutant diptheria toxin); and a *Neisseria* outer membrane protein. Oligo- and polysaccharides from GBS might be used in a vaccine. Oligosaccharide- and polysaccharide-protein conjugates alter the immunological properties of the polysaccharide or oligosaccharide and may improve the immune response.

An adjuvant may be used to enhance the immune response to a vaccine containing the *spb1* and/or *spb2* gene products. An adjuvant is any substance that enhances the immune response to an antigen. Without being bound by any particular theory, adjuvants may act by retaining the antigen locally near the site of administration to produce a depot

effect, facilitating the slow, sustained release of the antigen to cells of the immune system. Adjuvants may also attract cells of the immune system. Aluminum hydroxide and aluminum phosphate (collectively and commonly referred to as "alum") are routinely used as adjuvants in human and veterinary vaccines. Currently, alum is the only adjuvant licensed for human use, although a number of experimental adjuvants are being tested.

The *spb1* and/or *spb2* gene(s) may also be introduced into a mammal using either naked DNA or other gene therapy techniques to induce an immune response against virulent GBS.

All publications, patents, and patent applications cited in this application are hereby incorporated by reference. U.S. Patent Application Serial No. 60/140,084 is hereby incorporated by reference in its entirety.

6. EXAMPLES

The following examples are given to illustrate several embodiments which have been made within the scope of the present invention. It is to be understood that these examples are neither comprehensive nor exhaustive of the many types of embodiments which can be prepared in accordance with the present invention.

Example 1 - Isolation of the *spb1* and *spb2* genes

Bacterial factors that contribute to the increased virulence of III-3 strains can be identified by characterizing the differences between the genetic composition of III-3 and III-2 strains. Such genetic differences will be found in the bacterial chromosomes since these strains do not contain plasmids. Takahashi et al., *supra*.

To identify genes present in virulent type III-3 GBS strains and not in the avirulent type III-2 strains, a modification of the technique described by Lisitsyn et al., *Science* **259**:946-951 (1993), was used. High molecular weight genomic DNA from an invasive RDP type III-3 GBS strain (strain 874391) and a colonizing ("avirulent") RDP type III-2 strain (strain 865043) were prepared by cell lysis with mutanolysin and Proteinase K digestion. Y. Nagano et al., *supra*. For genetic subtraction, genomic DNA from both strains was digested with *Taq* I. *Taq* I-digested DNA from the virulent strain was mixed with two complementary oligonucleotides, TaqA (5'-CTAGGTGGA-TCCTTCGGCAAT-3' (SEQ ID NO: 5)) and TaqB (5'-CGATTGCCGA-3' (SEQ ID NO: 6)), heated to 50°C for 5 minutes, then allowed to cool slowly to 16°C in T4 ligase

buffer. Oligonucleotides were ligated to the virulent strain DNA by incubation with 20 units of T4 ligase at 16°C for 12 hours. After ligation, 500 ng of DNA from the virulent strain, with ligated linkers, and 40 μg of DNA from the avirulent strain, without linkers, was mixed together, denatured by heating, and hybridized at 68°C for 20 hours.

Ten percent of the resulting hybridization mixture was incubated with *Taq* DNA polymerase and dNTPs to fill in the ends of annealed virulent strain DNA. The hybridized DNA was amplified by *Taq* DNA polymerase for 10 cycles using the TaqA oligonucleotide as the forward and reverse amplification primer. After amplification, single stranded products remaining after amplification were digested with mung bean nuclease. Twenty percent of the resulting product was then reamplified for 20 cycles. This process of subtraction followed by PCR amplification results in enhanced amplification of DNA segments from the III-3 strains that do not hybridize with DNA segments from the III-2 strains.

A total of four cycles of subtraction and amplification were carried out using successively smaller quantities of III-3 specific PCR products. Two pairs of oligonucleotides were used for subtraction, which were alternated with successive rounds of subtraction-amplification. The oligos were TaqA (SEQ ID NO: 5) and TaqB (SEQ ID NO: 6) (the first pair) and TaqE (5'-AGGCAACTGTGCTAACCGAGGGAAT-3' (SEQ ID NO: 7)) and TaqF (5'-CGATTCCCTCG-3' (SEQ ID NO: 8)) (the second pair). The final amplification products were ligated into pBS KS+ vector and transformed into competent XL1-Blue strain *E. coli*. Thirteen clones were randomly selected for analysis. Cross hybridization studies of the 13 inserts revealed that 6 were unique. These probes were used in slot and dot blot experiments to determine whether subtraction was successful and to identify probes hybridizing with all III-3 strains. Each of the 6 unique probes hybridized with the parental III-3 virulent strain, while none of the probes hybridized with the avirulent III-2 strains. Three of the amplified sequence tags (clones 1, 3 and 11) hybridized with genomic DNA from all 62 type III-3 isolates, but did not hybridize with DNA prepared from the III-2 and III-1 isolates.

Figure 2 illustrates a dot blot hybridization of type III GBS genomic DNA hybridized with a clone 1 probe. 10 μg of genomic DNA from each of 62 type III GBS strains was transferred to nylon membrane. Radiolabeled clone 1 probe hybridized with DNA from all III-3 strains (rows A-D) including the original type III-3 strain (well E-1).

The probe failed to hybridize with DNA from III-2 strains (F1-F10, G1-G7) including the original strain used in the subtraction hybridization (well E10) and III-1 strains (wells H1-H3). The same pattern of hybridiztion was observed using clone 3 and 11 probes. These data demonstrate the feasibility of identifying genes unique to III-3 strains by this method of PCR-based subtractive hybridization, and further support the validity of the RDP typing for identifying genetically-related type III GBS.

The three GBS type III-3-specific sequence tags are short (130-360 bp). To obtain additional sequence information, a genomic GBS III-3 library was constructed. High molecular weight GBS genomic DNA was partially digested with *Bgl* II and cloned into λ FIX II phage vector. Phage were packaged and the library, consisting of 1.7×10^5 recombinant phage containing inserts with a mean size of about 18 kb (totaling approximately 3×10^9 bp), was amplified once. Multiple plaques hybridizing with each of the III-3 GBS-specific probes were purified for further characterization.

Three overlapping genomic clones hybridizing with probe 1 were identified, with approximate sizes of 9, 22, and 23 kb. Since the boundaries of GBS III-3 specific DNA are not known, smaller fragments were subcloned and the DNA was verified present in virulent GBS strains before proceeding with further characterization. The first segment examined is a 6.4 kb *Sal* I-*Bg*l II fragment. This genomic DNA is present in all RDP type III-3 strains and in none of 17 RDP type III-2 strains.

Over 90% of this genomic DNA fragment has been sequenced and found to contain 5 open reading frames ("ORFs"). Three of these are likely to be authentic genes. They feature ATG start sites, are preceded by potential ribosomal binding sites and, in two cases, stop codons are followed by palindromic sequences that may represent transcriptional terminators. They are relatively short, however, and do not show significant homology at the nucleic acid or amino acid level with sequences registered with Genbank or the available bacterial genome databases.

The two other ORFs appear to be more obvious candidates for virulence genes. The *spb1* gene includes a 1509 bp ORF that is preceded by a potential ribosomal binding site 10 bases upstream from an ATG start codon. The predicted protein (502 amino acids and Mr 53,446) has the characteristics of a cell wall-bound protein. The N-terminus of the predicted protein is a hydrophilic, basic stretch of 6 amino acids followed by a 23 amino acid hydrophobic, proline-rich core, consistent with a signal peptide. The

hydrophilic mature protein terminates in a typical LPXTG (SEQ ID NO: 9) domain that immediately precedes a hydrophobic 20 amino acid core and a short, basic hydrophilic terminus.

The nucleotide sequence is not homologous to sequences of other known bacterial genes. The translated amino acid sequence, however, shares segmental homology with a number of characterized proteins, including the fimbrial type 2 protein of *Actinomyces naeslundii* (27% identity over 350 amino acids) and the fimbrial type 1 protein of *Actinomyces viscosus* (25% homology over 420 amino acids), the T6 surface protein of *S. pyogenes* (23% identity over 359 amino acids), and the *hsf* (27% identity over 260 amino acids) and HMW1 adhesins (25% identity over 285 amino acids) of *Haemophilus influenzae*. *See* M.K. Yeung & J.O. Cisar, *J Bacteriol* **172**:242-2468 (1990); O. Schneewind, et al., *J Bacteriol* **172**:3310-3317 (1990); J.W. St. Geme III, et al., *J Bacteriol* **178**:6281-6287 (1996); J.W. St. Geme III, *Infect Immun* **62**: 3881-3889 (1994). The function of the *S. pyogenes* T6 protein is unknown. Each of the other homologues plays a role in bacterial adhesion and/or invasion.

The second ORF, *spb2*, terminates 37 bp upstream from *spb1* and is in the same transcriptional orientation. This 1692 bp ORF has a deduced amino acid sequence of 563 residues and Mr 64,492. It shares 50.5% nucleic acid identity and 20.7% amino acid identity with *spb1*. Conservation is highest in the carboxy-terminal regions, including a shared LPSTGG (SEQ ID NO: 10) motif. In contrast to *spb1*, *spb2* does not have an obvious signal sequence. Its secretion may be mediated by carboxy-terminal recognition sequences or by accessory peptides. T. Michiels, et al., *Infect Immun* **58**:2840-2849 (1990). The deduced amino acid sequence of *spb2* is also homologous with *S. pyogenes* T6 and *Actinomyces naeslundii* proteins, and to *Listeria monocytogenes* internalin A (22% identity over 308 amino acids)—again, proteins important in adhesion and invasion.

Neither of the predicted gene products has the repetitive structure of previously described GBS surface proteins such as the C and Rib proteins or of *L. monocytogenes* internalin family members. L.C. Madoff et al., *Infect Immun* **59**:2638-2644 (1991); J. Gaillard, et al., *Cell* **65**: 1127-1141 (1991). Hybridization of the originating strain 874391 genomic DNA with an *spb1* probe under low stringency conditions identifies a single band in *EcoR* I, *Sal* I and *Sst* I digests (data not shown), suggesting that a single

copy of *spb1* is present in this strain and that *spb1* is not a member of a significantly homologous "family" of genes.

Example 2 - Adherence and Invasion Assays Using *spb1*⁻ Bacteria

Genomic subtraction identified a 1509 bp ORF, *spb1*, which is present in virulent RDP type III-3 GBS and not in RDP type III-2 strains. The predicted 53 kD protein product has the characteristics of a typical gram positive cell-wall bound protein. The nucleic acid sequence of *spb1* is not homologous to sequences of other known bacterial genes, however the translated amino acid sequence shares segmental homology with several characterized adhesins, including *Actinomyces* fimbrial proteins and *H. influenzae* HMW1, suggesting that Spb1 might contribute to GBS adhesion or invasion. A *spb1*$^-$ isogenic deletion mutant GBS strain was created by homologous recombination and the ability of the *spb1*$^-$ mutant to adhere to and invade A549 respiratory epithelial cells was determined. Compared to the wild type strain, the number of *spb1*$^-$ bacteria adherent to A549 monolayers was reduced by 60.0% ($p<0.01$) and the number of intracellular invading bacteria was reduced by 53.6% ($p<0.01$). Without being bound by any particular theory, it appears that Spb1 may contribute to the pathogenesis of GBS pneumonia and bacterial entry into the bloodstream.

CLAIMS:

1. An isolated nucleic acid molecule comprising nucleotides which code for the amino acid sequence of SEQ ID NO: 2.

2. A recombinant vector comprising the nucleic acid molecule of claim 1.

3. The recombinant vector of claim 2, wherein said recombinant vector is a plasmid.

4. The recombinant vector of claim 2, wherein said recombinant vector is a prokaryotic or eukaryotic expression vector.

5. The recombinant vector of claim 2, wherein the nucleic acid molecule is operably linked to a heterologous promoter.

6. A host cell comprising the nucleic acid molecule of claim 1.

7. The host cell of claim 6, wherein the host cell is a eukaryotic host cell.

8. The host cell of claim 6, wherein the host cell is a prokaryotic host cell.

9. An isolated nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1.

10. A recombinant vector comprising the nucleic acid molecule of claim 9.

11. The recombinant vector of claim 10, wherein said recombinant vector is a plasmid.

12. The recombinant vector of claim 10, wherein said recombinant vector is a prokaryotic or eukaryotic expression vector.

13. The recombinant vector of claim 10, wherein the nucleic acid molecule is operably linked to a heterologous promoter.

14. A host cell comprising the nucleic acid molecule of claim 9.

15. The host cell of claim 14, wherein the host cell is a eukaryotic host cell.

16. The host cell of claim 14, wherein the host cell is a prokaryotic host cell.

17. An isolated nucleic acid molecule comprising nucleotides which code for the amino acid sequence of SEQ ID NO: 4

18. A recombinant vector comprising the nucleic acid molecule of claim 17.

19. The recombinant vector of claim 18, wherein said recombinant vector is a plasmid.

20. The recombinant vector of claim 18, wherein said recombinant vector is a prokaryotic or eukaryotic expression vector.

21. The recombinant vector of claim 18, wherein the nucleic acid molecule is operably linked to a heterologous promoter.

22. A host cell comprising the nucleic acid molecule of claim 17.

23. The host cell of claim 22, wherein the host cell is a eukaryotic host cell.

24. The host cell of claim 22, wherein the host cell is a prokaryotic host cell.

25. An isolated nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 3.

26. A recombinant vector comprising the nucleic acid molecule of claim 25.

27. The recombinant vector of claim 26, wherein said recombinant vector is a plasmid.

28. The recombinant vector of claim 26, wherein said recombinant vector is a prokaryotic or eukaryotic expression vector.

29. The recombinant vector of claim 26, wherein the nucleic acid molecule is operably linked to a heterologous promoter.

30. A host cell comprising the nucleic acid molecule of claim 25.

31. The host cell of claim 30, wherein the host cell is a eukaryotic host cell.

32. The host cell of claim 30, wherein the host cell is a prokaryotic host cell.

33. A method of immunizing a mammal against Group B streptococci infection, said method comprising administering to the mammal a vaccine comprising an immunologically effective amount of a recombinantly produced protein comprising the amino acid sequence of SEQ ID NO: 2.

34. The method of claim 33, wherein the vaccine further comprises an adjuvant.

35. The method of claim 34, wherein the adjuvant comprises alum.

36. The method of claim 33, wherein the vaccine further comprises an immunologically effective amount of a recombinantly produced protein comprising the amino acid sequence of SEQ ID NO: 4.

37. The method of claim 36, wherein the vaccine further comprises an adjuvant.

38. A method of immunizing a mammal against Group B streptococci infection, said method comprising administering to the mammal a vaccine comprising an immunologically effective amount of a recombinantly produced protein comprising the amino acid sequence of SEQ ID NO: 4.

39. The method of claim 38, wherein the vaccine further comprises an adjuvant.

40. The method of claim 39, wherein the adjuvant comprises alum.

41. A diagnostic method for determining whether a mammal is infected or colonized by virulent Group B streptococci (GBS), said method comprising the steps of:

(a) collecting a bodily fluid or culture from the mammal;

(b) analyzing the bodily fluid or culture for the presence or absence of one or more gene products specific to type III-3 GBS;

wherein the presence of one or more gene products specific to type III-3 GBS indicates infection or colonization by virulent GBS.

42. The diagnostic method of claim 41, wherein the one or more gene products specific to type III-3 GBS comprise a protein, said protein comprising the amino acid sequence of SEQ ID NO: 2.

43. The diagnostic method of claim 41, wherein the one or more gene products specific to type III-3 GBS comprise a protein, said protein comprising the amino acid sequence of SEQ ID NO: 4.

44. The diagnostic method of claim 41, wherein the one or more gene products specific to type III-3 GBS comprise a first protein comprising the amino acid sequence of SEQ ID NO: 2 and a second protein comprising the amino acid sequence of SEQ ID NO: 4.

45. The diagnostic method of claim 41, wherein the mammal is a human.

46. The diagnostic method of claim 41, wherein the bodily fluid or culture is a vaginal or rectovaginal culture.

47. The diagnostic method of claim 41, wherein the bodily fluid or culture is a throat culture.

48. The diagnostic method of claim 41, wherein the bodily fluid or culture is blood, serum, amniotic fluid, cerebrospinal fluid, or joint fluid.

49. The diagnostic method of claim 41, wherein the analysis step comprises using polymerase chain reaction (PCR) to identify the presence or absence of one or more gene products specific to type III-3 GBS.

50. The diagnostic method of claim 49, wherein the analysis step comprises using PCR to determine the presence or absence of the *spb1* gene product.

51. The diagnostic method of claim 49, wherein the analysis step comprises using PCR to determine the presence or absence of the *spb2* gene product.

52. The diagnostic method of claim 41, wherein the analysis step comprises using antibodies to identify the presence or absence of one or more gene products specific to type III-3 GBS.

53. The diagnostic method of claim 52, wherein the antibodies are monoclonal antibodies.

54. The diagnostic method of claim 53, wherein the monoclonal antibodies are specific for the *spb1* gene product.

55. The diagnostic method of claim 53, wherein the monoclonal antibodies are specific for the *spb2* gene product.

56. An isolated and purified protein comprising the amino acid sequence of SEQ ID NO: 2.

57. A vaccine for immunizing a mammalian host against virulent Group B streptococci infection, said vaccine comprising the protein of claim 56.

58. The vaccine of claim 57, further comprising an adjuvant.

59. The vaccine of claim 58, wherein the adjuvant comprises alum.

60. The vaccine of claim 57, wherein the protein is conjugated to a bacterial polysaccharide or oligosaccharide.

61. An isolated and purified protein comprising the amino acid sequence of SEQ ID NO: 4.

62. A vaccine for immunizing a mammalian host against virulent Group B streptococci infection, said vaccine comprising the protein of claim 61.

63. The vaccine of claim 62, further comprising an adjuvant.

64. The vaccine of claim 63, wherein the adjuvant comprises alum.

65. The vaccine of claim 62, further comprising an isolated and purified protein comprising the amino acid sequence of SEQ ID NO: 2.

66. The vaccine of claim 65, further comprising an adjuvant.

67. The vaccine of claim 62, wherein the protein is conjugated to a bacterial polysaccharide or oligosaccharide.

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(54) Title: ISOLATED GENES FROM VIRULENT GROUP B *STREPTOCOCCUS AGALACTIAE*

(57) Abstract: The present invention relates to the identification and prevention of infections by virulent forms of Group B streptococci. Disclosed herein is the identification of two genes, *spb1* and*spb2*, that are specific to virulent type III-3 GBS. Also disclosed herein are diagnostic methods for detecting virulent GBS infections and methods of immunizing a mammal against these bacteria.

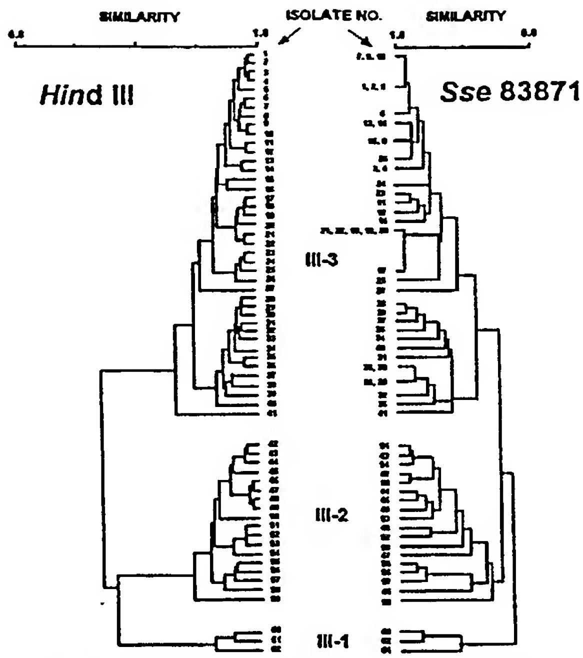

Figure 1. Type III GBS RDP types. Comparison of *Hind*III and *Sse*83871 RDP typing of 62 type III GBS isolates from Salt Lake City and Tokyo. Isolates were classified into types based on the similarity of the restriction digest patterns produced by *Hind*III or *Sse*83871 digestion of chromosomal DNA. The two methods divided the isolates into RDP types containing exactly the same isolates: III-3 contains isolates 1 - 41, III-2 contains isolates 42 - 59, and III-1 contains isolates 60-62.

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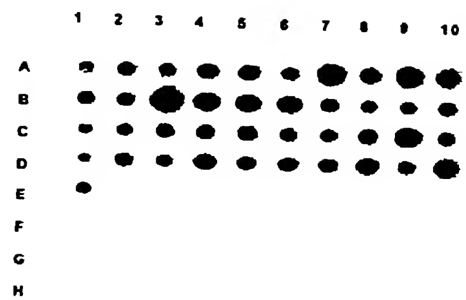

Figure 2. RDP type III-3 specific probes. Dot blot hybridization of probe 1 with genomic DNA isolated from type III GBS. 10 ug of genomic DNA from each of 62 type III GBS strains was transferred to nylon membrane. Radiolabeled probe 1 hybridized with DNA from all III-3 strains (rows A-D) including the original type III-3 strain (well E-1). The probe failed to hybridize with DNA from III-2 strains (F1-F10, G1-7) including the original strain used in the subtraction hybridization (well E 10) and III-1 strains (wells H1-3). The same pattern of hybridization was observed using clone 3 and 11 probes.

PATENT APPLICATION
Docket No.: 1321.2.29.1

DECLARATION, POWER OF ATTORNEY AND PETITION

We, Elisabeth E. Adderson and John F. Bohnsack, declare: that we are citizens of the United States of America; that our residences and post office addresses are St. Jude Children's Research Hospital, 332 N. Lauderdale, Rm D2038, Memphis, Tennessee 38105 and Pediatric Administration, University of Utah School of Medicine, 30 North Medical Drive, Room 2A152, Salt Lake City, Utah 84112, respectively; that we verily believe we are the original, first, and joint inventors of the subject matter of the invention or discovery entitled GENES FROM VIRULENT GROUP B STREPTOCOCCUS AGALACTIAE, for which a patent is sought and which is described and claimed in the specification filed in the United States Patent and Trademark Office as Serial No. 10/009,254 on December 10, 2001; that we have reviewed and understand the contents of the above-identified specification, including the claims; and that we acknowledge the duty to disclose all information known to us to be material to patentability as defined in Section 1.56 of Title 37 of the Code of Federal Regulations.

We hereby claim the benefit under Section 119 of Title 35 of the United States Code of the earlier filed pending application, Serial No. 60/140,084, filed June 21, 1999; and, insofar as the subject matter of each of the claims of this application is not disclosed in the earlier filed pending application in the manner provided by the first paragraph of Section 112 of Title 35 of the United States Code, we acknowledge the duty to disclose material information, as defined in Section 1.56(a) of Title 37 of the Code of Federal Regulations, which occurred between the filing date of the earlier filed application and the filing date of this application.

We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

We hereby appoint as our attorneys and/or patent agents those individuals associated with U.S. Patent and Trademark Office Customer No. 21552 with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. All correspondence and telephonic communications should be directed to Eric M. Barzee at the address associated with Customer No. 21552.

Wherefore, we pray that Letters Patent be granted to us for the invention or discovery described and claimed in the foregoing specification and claims, declaration, power of attorney, and this petition.

Signed at Salt Lake City (City), UT (State), this 17 day of May, 2002.

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